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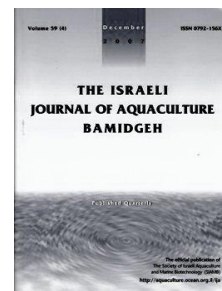
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## **Pigmentary and Zootechnical Responses of Juvenile *Litopenaeus vannamei* (Boone, 1931) Maintained on Diets Supplemented with Xanthophylls of Marigold *Tagetes erecta* Flowers**

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### **Abstract**

Practical diets supplemented with 75 or 150 ppm xanthophylls (75% zeaxanthin, 15% lutein) industrially extracted from marigold (*Tagetes erecta* L.) flowers increased the astaxanthin and total carotenoid concentrations in juvenile *Litopenaeus vannamei*, compared to shrimp fed a practical control diet. Our results paralleled or exceeded those obtained with a diet containing 75 ppm supplementary synthetic astaxanthin. The post-feeding astaxanthin concentration accounted for more than 84% of the total carotenoid concentration in shrimp fed either diet, while beta-carotene, zeaxanthin, lutein, and other non-identifiable carotenoids comprised a minority of the total concentration. That this was seen in both the tail exoskeleton and abdominal muscle indicates that *L. vannamei* can metabolize precursor xanthophylls to produce astaxanthin. In most cases, more than 60% of the astaxanthin was esterified. In general, survival improved in shrimps fed the supplemented diets compared to those fed the control diet. There were no differences in growth.

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## Introduction

Astaxanthin is the most abundant carotenoid in the body of some species of penaeid shrimp, which are unable to synthesize the compound *de novo* but are known to metabolize precursors such as beta-carotene and lutein into astaxanthin (Meyers and Latscha, 1997). This carotenoid has the highest antioxidant activity of any known naturally-occurring compound. Its capacity to chelate toxic products of cellular metabolism is higher than that of vitamin E (Miki, 1991). Astaxanthin inhibits the peroxidation of mitochondrial lipids, and its ability to span the phospholipid bilayer contributes to the stability of the cell membrane (Goto et al., 2001).

When diets of some cultivated penaeid species are enriched with natural or synthetic astaxanthin, the oxycarotenoid concentration in the shrimp body increases (Yamada et al., 1990; Chien and Jeng, 1992; Petit et al., 1998). This in turn improves survival and growth due to the enhancement of various physiological responses (Chien and Shiau, 2005; Flores et al., 2007).

Since astaxanthin is expensive (Boonyaratpalin et al., 2001), researchers have experimented with different natural products that are rich in astaxanthin precursor carotenoids. These products can be obtained from marigold flowers (Vernon-Carter et al., 1996; Göçer et al., 2006; Jha et al., 2012), chili pepper (Arredondo-Figueroa et al., 2003), and microalgae (Liao et al., 1993; Díaz et al., 2011). Despite promising results, most of these products are hand-made or experimental items, and their use on an industrial scale is currently unfeasible.

Commercial products from processed marigold (*Tagetes erecta* L.) flowers are used worldwide by the poultry and nutraceutical industries. Such products contain a high concentration of a stable mix of lutein and zeaxanthin, which are precursors of astaxanthin. When included in vegetable oil, they are easy to integrate in animal feed pellets (Del Villar-Martínez et al., 2007). Thus, they could be worthwhile options for aquaculture if their effectiveness as pigmentation agents for shrimp species is confirmed.

Diets supplemented with oleoresin from marigold flowers cause an increase in total carotenoid concentration in the body of *Litopenaeus vannamei* (Arredondo-Figueroa et al., 2003; Vernon-Carter et al., 1996). This report describes two experimental trials in which diets supplemented with zeaxanthin extracts obtained from marigold flowers resulted in an increase of astaxanthin concentration and survival in juvenile *L. vannamei*.

## Materials and Methods

**Diets.** Xanthophyll extract in vegetable oil was prepared by industrial processing of marigold *T. erecta* flowers (Hi Zea® Industrial Orgánica S.A. de C.V.; 30000 ppm xanthophylls: 75% zeaxanthin and 20% lutein, both as short chain esters, and 5% other xanthophylls that were not astaxanthin). The xanthophyll and synthetic astaxanthin (Carophyll Pink Roche® 8%) were added to a non-commercial practical diet used to feed experimental shrimp populations cultured in the Universidad de Sonora facilities (Table 1). Food pellets with different concentrations of carotenoid pigments based on this diet included: (a) the control diet containing 4 ppm carotenoids from the basal ingredients, i.e., no supplement, (b) a diet containing 75% xanthophylls from *T. erecta*, (c) a diet containing 150% xanthophylls from *T. erecta*, and (d) a diet containing 75% synthetic astaxanthin. The latter diet, used as a reference diet, was based on previously used astaxanthin concentrations (Yamada et al., 1990; Petit et al., 1998; Flores et al., 2007).

To make the food pellets, the wet mass of ingredients was extruded through a threaded ring with orifices of 3/32" in diameter using a meat grinder. The required portion of each pigment product was incorporated into the liquid ingredient mixture. After drying, the pellets were broken and sieved to obtain food particles of 2000-2500 µm, as required by the experimental protocol, and stored in dark, ambient conditions. Pellets were stable for at least 24 h in salt water. The proximal compositions of the pellets were analyzed by official methods (AOAC, 1995). The carotenoid contents of the pellets were analyzed as described below.

**Experiment 1.** The first experiment compared the carotenoid and astaxanthin concentrations in organisms fed diets supplemented with xanthophylls or synthetic

Table 1. Practical diet used to feed *Litopenaeus vannamei*.

Ingredient	%
Fishmeal <sup>1</sup>	30
Soybean meal <sup>2</sup>	25
Whole wheat meal	22
Shrimp meal <sup>3</sup>	10
Dibasic calcium phosphate <sup>4</sup>	3
Fish oil <sup>1</sup>	2
Vitamin premix <sup>5</sup>	1.8
Fish soluble additives <sup>1</sup>	1.7
Soybean oil	1
Sodium alginate <sup>6</sup>	1
Sodium hexametaphosphate <sup>4</sup>	1
Lecitin <sup>4</sup>	0.5
Mineral premix <sup>7</sup>	0.5
Cholesterol <sup>6</sup>	0.2
Choline chloride <sup>6</sup>	0.2
Stay-C <sup>8</sup>	0.1

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astaxanthin. Four treatments were studied in triplicate in a raceway (30 x 3 x 0.7 m) with twelve cages (1 x 1 x 1 m) inside a greenhouse. The cages were made of wood and plastic nets (Vexar; 13 x 13 mm mesh); the floor and roof were covered with a black polyethylene film. From an F1 population of at least 25,000 shrimp intensively raised in an adjacent raceway, 50 organisms (1.6±0.2 g) were collected and placed in each cage. They were fed the control diet for one week and then one of four diets for four additional weeks. The shade in the greenhouse and daily brushing of all cage and raceway surfaces prevented fouling and, therefore, the shrimp had no other source of food.

Survival was evaluated by direct count every week. The mean individual weight was estimated from a sample of ten organisms from each replicate. The daily feed ration equalled 10% of the biomass, divided into three equal portions given every 8 h. Unconsumed food, feces, and exuviae were retrieved daily.

Salt water (38 g/l) was pumped from a coastal well through a sand filter system and a longitudinal manifold placed on the raceway wall. Daily water exchange was 100% and the mean water temperature was 28±1°C. At the end of the experiment, two shrimp in the intermolt stage from each experimental unit were collected (six specimens per treatment) to determine the mean carotenoid concentration in each group. Extracts of abdominal muscle and tail exoskeleton were analyzed by spectrophotometric procedures as described below. One pooled sample per treatment was analyzed to assess the carotenoid profile and free, monoester, and diester astaxanthin concentrations.

**Experiment 2.** In the first experiment, carotenoid profiles and astaxanthin concentrations were analyzed in pooled samples. The second experiment took individual variations into account by analyzing individual shrimps. There were three treatments with five replicates each: (a) a control diet as described for

experiment 1, (b) a diet containing 75% xanthophylls from *T. erecta*, and (c) a diet containing 150% xanthophylls from *T. erecta*. Fifteen groups of 50 F1 offspring (2.5±0.3 g) of the same initial broodstock used in experiment 1 were placed in experimental cages under the same conditions as described in experiment 1. At the end of the 4-week experimental period, three shrimps in the intermolt stage were collected from each cage (fifteen per treatment) and the mean concentrations of total carotenoids and free, monoester, and diester astaxanthin in the abdominal muscle and tail exoskeleton were determined for each shrimp. Carotenoids were identified and quantified by spectrophotometric analysis and high pressure liquid chromatography (HPLC) as described by Boonyaratpalin et al. (2001) and Plank et al. (2002).

**Total carotenoid concentrations.** The head was removed from each specimen and discarded. The tail exoskeleton and abdominal muscle were separated and macerated in a porcelain mortar to a fine homogenous paste. Fractions of a known weight were placed in flasks and carotenoids were extracted first with acetone, then by hexane, and stored in the dark. The same procedure was repeated until the solutions turned clear. These were then transferred to a flask to which crystalline Na<sub>2</sub>SO<sub>4</sub> anhydrous was added, diluted in hexane, and the final volume was measured. Total carotenoid concentrations were

analyzed spectrophotometrically from the peak absorbance of 470 nm using the specific extinction coefficient  $E^{1\%}_{1\text{cm}} = 2100$  (Weber, 1988). Astaxanthin was the main pigment and its concentration was determined as:  $\mu\text{g carotenoids}/\text{W} = (\text{Abs} \times \text{DF} \times \text{V}) / (2100 \times \text{W})$ , where W = wt of sample in g, Abs = absorbance of hexane solution at 470 nm, DF = dilution factor, V = volume, and 2100 = astaxanthin absorptivity in hexane.

**Carotenoid profile and astaxanthin analysis.** Twenty ml of the hexane solution from each sample were evaporated to dryness under ambient nitrogen and the residue was diluted in 2 ml of the mobile phase (86%hexane:14%acetone by volume) and filtered through a 0.2- $\mu\text{m}$  membrane. Chromatographic analysis of each sample was performed with a Beckman System Gold model 126, equipped with two pumps, and a Diode Array Detector model 168, with a pre-column Beckman (Si60; 5  $\mu\text{m}$ ; 100  $\times$  4.6 mm) and a Phenomenex column (Si60 Å; 5  $\mu\text{m}$ ; 250  $\times$  4.6 mm) treated with  $\text{H}_3\text{PO}_4$ . The volume of the mobile phase was 10  $\mu\text{l}$  and the flow rate was adjusted to 1.5 ml/min. The carotenoids were detected at 470 nm and identified by co-injecting known standards (Industrial Orgánica S.A. de C.V., Monterrey, N.L.) and comparing their retention times and absorption spectra. Pigments were quantified by the internal normalization technique in which the analyte area is compared to the total area of all peaks present in a sample (Britton, 1995). Pigments in pellets were analyzed by the same procedures.

**Statistical analysis.** One-way analysis of variance (ANOVA) was applied to determine the statistical significance ( $p < 0.05$ ) of the differences in survival, mean final weight, and pigment concentrations. When necessary, Newman-Keuls tests were used to make pairwise comparisons between treatments (Zar, 1999). Statistical analyses were performed using the software Sigma-Stat 3.5 (SISTAT).

## Results

**Diet analysis.** There were no significant differences between diets in protein ( $37 \pm 0.65\%$ ), fat ( $5.5 \pm 0.48\%$ ), fiber ( $4.1 \pm 0.45\%$ ), ash ( $6 \pm 0.2\%$ ), or moisture ( $6.15 \pm 0.12\%$ ). After drying, the carotenoid content decreased by 2-4% in the supplemented diets, but the proportions of zeaxanthin and lutein remained the same, 75% and 20% respectively.

**Experiment 1.** Survival was significantly higher in shrimp fed the enriched diets than in shrimp fed the control diet but there were no significant differences in mean final weight (Table 2). In all analyzed specimens, the mean carotenoid concentration was higher in the tail exoskeleton than in the eviscerated abdominal muscle. The pigment profile was the same for all groups and astaxanthin was the main carotenoid in all samples of abdominal muscle and tail exoskeleton, representing 85% of the total (Fig. 1). No other pigments were identified. Except for the pooled abdominal muscle sample of shrimp fed the astaxanthin diet, esterified forms accounted for over 60% of the total astaxanthin concentration.

**Experiment 2.** As in experiment 1, survival was higher in groups fed the enriched diets than in

Table 2. Responses of *Litopenaeus vannamei* fed diets containing 75 or 150 ppm xanthophyll of marigold flowers, or 75 ppm of synthetic astaxanthin, or a control diet containing neither supplement.

	Diet			
	Control	Xanthophyll 75 ppm	Xanthophyll 150 ppm	Astaxanthin 75 ppm
Initial no.	50	50	50	50
Final no.	37.7 $\pm$ 1.5 <sup>a</sup>	43.6 $\pm$ 1.1 <sup>b</sup>	47.6 $\pm$ 0.5 <sup>b</sup>	44.6 $\pm$ 1.1 <sup>b</sup>
Survival (%)	75.5 <sup>a</sup>	87 <sup>b</sup>	95 <sup>b</sup>	89.2 <sup>b</sup>
Initial wt (g)	1.64 $\pm$ 0.2	1.64 $\pm$ 0.2	1.64 $\pm$ 0.2	1.64 $\pm$ 0.2
Final wt (g)	4.22 $\pm$ 0.7	4.76 $\pm$ 0.9	4.90 $\pm$ 1.0	4.40 <sup>a</sup> $\pm$ 0.8
<b>Pigments in abdominal muscle</b>				
Total carotenoids ( $\mu\text{g/g}$ ) <sup>1</sup>	16.0 $\pm$ 0.9 <sup>a</sup>	17.26 $\pm$ 2.0 <sup>a</sup>	20.30 $\pm$ 1.8 <sup>b</sup>	17.59 $\pm$ 1.5 <sup>a</sup>
Total astaxanthin ( $\mu\text{g/g}$ ) <sup>2</sup>	15.2	17.0	20.5	15.8
Free astaxanthin (% total)	39.1	37.2	34.9	54.3
Mono+diester astaxanthin (% total)	60.9	62.8	65.1	45.7
<b>Pigments in tail exoskeleton</b>				
Total carotenoids ( $\mu\text{g/g}$ ) <sup>1</sup>	74.1 $\pm$ 4.4 <sup>a</sup>	87.5 $\pm$ 8.3 <sup>b</sup>	89.4 $\pm$ 5.5 <sup>b</sup>	86.32 $\pm$ 7.0 <sup>b</sup>
Total astaxanthin ( $\mu\text{g/g}$ ) <sup>2</sup>	68.3	81.9	79.3	84.1
Free astaxanthin (% total)	37.9	35.2	33.2	36.0
Mono+diester astaxanthin (% total)	62.1	64.8	66.8	63.9

Means in a row with different superscripts significantly differ ( $p < 0.05$ ).

<sup>1</sup> Mean value of six individual samples per treatment

<sup>2</sup> Value of one pool of samples per treatment.



groups fed the control diet and there were no significant differences in the mean weights (Table 3). The highest carotenoid concentration was obtained in the tail exoskeleton of shrimp fed the 150 ppm xanthophyll diet. The increase of total carotenoid in groups fed the 75 ppm and 150 ppm xanthophyll diets corresponds to the increase in astaxanthin concentration. The same statistical trends were observed in the total carotenoid and astaxanthin concentrations in the abdominal muscle. Astaxanthin represented over 84% of the total carotenoid concentration in both the abdominal muscle and the tail exoskeleton of each analyzed specimen, with beta-carotene, lutein, and zeaxanthin representing small portions of the pigment profile (Fig. 2). In each case, esterified forms of astaxanthin accounted for over 61% of the total astaxanthin concentration.

Table 3. Responses of *Litopenaeus vannamei* fed diets containing 75 or 150 ppm xanthophyll of marigold flowers, or a control diet containing no supplemental xanthophyll (mean values of 15 individuals per treatment).

	Diet		
	Control	Xanthophyll 75 ppm	Xanthophyll 150 ppm
Initial no.	50	50	50
Final no.	40.8±1.9 <sup>a</sup>	47.6±0.8 <sup>b</sup>	48.6±1.1 <sup>b</sup>
Survival (%)	81.6 <sup>a</sup>	95.2 <sup>b</sup>	97.2 <sup>b</sup>
Initial wt (g)	2.5±0.3	2.5±0.3	2.5±0.3
Final wt (g)	6.7±1.1	6.5±1.3	6.7±0.9
<i>Pigments in abdominal muscle</i>			
Total carotenoids (µg/g)	13.1±2.7 <sup>a</sup>	15.7±1.8 <sup>a</sup>	21.6±5.8 <sup>b</sup>
Total astaxanthin (µg/g)	10.4±2.7 <sup>a</sup>	14.1±1.7 <sup>a</sup>	20.6±5.9 <sup>b</sup>
Free astaxanthin (% total)	38.1	27.1	29.3
Mono+diester astaxanthin (% total)	61.9	72.9	70.7
<i>Pigments in tail exoskeleton</i>			
Total carotenoids (µg/g)	76.3±15.6 <sup>a</sup>	88.6±14.8 <sup>a</sup>	100.5±11.1 <sup>b</sup>
Total astaxanthin (µg/g)	71.9±13.0 <sup>a</sup>	79.5±12.9 <sup>a</sup>	94.8±9.7 <sup>b</sup>
Free astaxanthin (% total)	31.8	26.4	27.9
Mono+diester astaxanthin (% total)	68.2	73.6	72.1

Means in a row with different superscripts significantly differ ( $p < 0.05$ ).

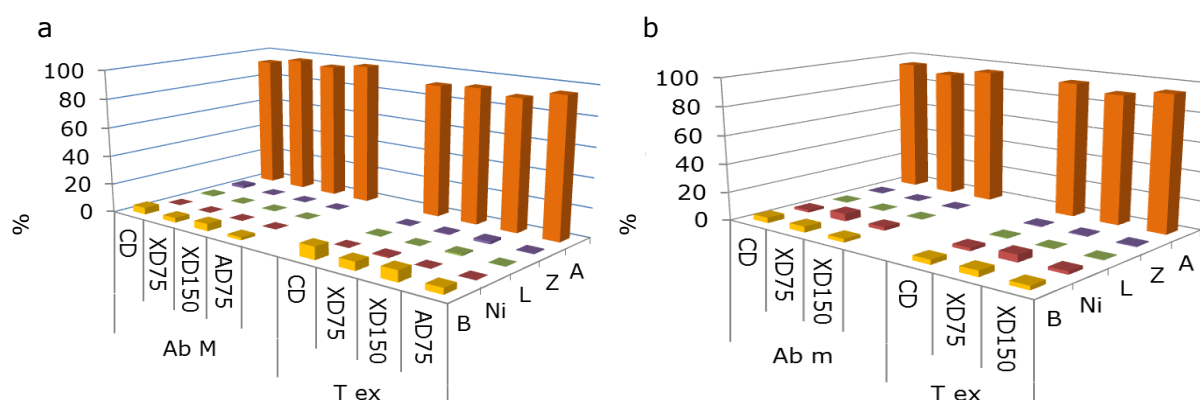


Fig. 1. Pigment profiles in abdominal muscle (Ab m) and tail exoskeleton (T ex) of juvenile white shrimp *Litopenaeus vannamei* fed diets containing an unsupplemented control (CD), 75 ppm xanthophyll (XD75), 150 ppm xanthophyll (XD150), or 75 ppm astaxanthin (AD75). Samples were pooled in (a) Experiment 1 and analyzed separately in (b) Experiment 2. B = betacarotene, Ni = no identified pigments, L = lutein, Z = zeaxanthin, A = astaxanthin.

## Discussion

The diets supplemented with natural zeaxanthin and lutein increased survival as well as total carotenoid and astaxanthin concentrations in *L. vannamei* to the same extent as the diet supplemented with synthetic astaxanthin. Natural and synthetic astaxanthin-supplemented diets have a positive effect on pigmentary response and survival in penaeid shrimp in experimental and commercial situations (Chien and Jeng, 1992; Kurmaly, 1993; Nègre-Sadargues et al., 1993; Darachai et al., 1998; Boonyaratpalin et al., 2001). Supplementing diets with astaxanthin may be more efficient than using natural or synthetic precursors because astaxanthin does not require metabolic transformation to facilitate its accumulation in shrimp tissues (Yamada et al., 1990; Latscha, 1991; Chien and Jeng, 1992; Niu et al., 2011). However, this study shows that xanthophylls from

marigold flowers are equally effective, similar to results in *P. monodon* using *Dunalliella salina* extracts rich in beta carotene (Boonyaratpalin et al., 2001). The high lutein and zeaxanthin concentrations in the supplemented diets and low concentrations in shrimp that consumed those diets show that the xanthophyll precursors were metabolized by the *L. vannamei* to form astaxanthin, which accumulated mainly in its esterified form. The pigment profile, in which astaxanthin predominated beta-carotene, zeaxanthin, and other carotenoids, agrees with observations in *P. monodon*, *P. indicus*, *P. japonicus*, and *M. dobsoni* (Nègre-Sadargues et al., 1993; Pan and Chien, 2004; Sachindra et al., 2005).

Factors controlling carotenoid absorption, transportation, and excretion between tissues in penaeids are not fully known (Boonyaratpalin et al., 2001). In some fish, xanthophyll esters are hydrolyzed in the digestive tract and re-esterified before they accumulate in the tissue (White et al., 2003). Dietary astaxanthin, beta-carotene, and canthaxanthin lead to the accumulation of mainly astaxanthin esters in *P. japonicus* and *P. monodon*, irrespective of the metabolic pathway and mechanisms of absorption and transport (Yamada et al., 1990; Chien and Jeng, 1992; Liao et al., 1993; Nègre-Sadargues et al., 1993; Boonyaratpalin et al., 2001).

Similar to results in our study of *L. vannamei*, survival increased in trials involving ecological challenge or optimal physical conditions, regardless of the carotenoid used to supplement the diet. This was demonstrated using synthetic astaxanthin for *P. monodon* and *P. japonicus* (Yamada, 1990; Chien and Jeng, 1992; Pan and Chien, 2004), natural astaxanthin for *P. monodon* (Darachai et al., 1998), and, in *L. vannamei*, carotenoid-rich extracts of red chili pepper (Arredondo-Figueroa et al., 2003) or lutein rich extracts of marigold flowers (Vernon-Carter et al., 1996). In experiments involving ecological challenge to investigate the efficiency of physiological responses, astaxanthin-supplemented diets resulted in decreased oxygen consumption under stress conditions in *Marsupeanus japonicus* (Chien and Shiau, 2005), higher resistance to osmotic shock in *P. monodon* (Merchie et al., 1998), and reduced oxygen consumption and ammonia production in *L. vannamei* acclimatized to low salinity (Flores et al., 2007).

In this study, there were no significant differences in growth between experimental groups fed different diets, similar to observations in *P. japonicus* and *P. monodon* (Boonyaratpalin et al., 2001; Pan et al., 2001). However, carotenoid supplemented diets had positive effects on growth in *P. japonicus* and *P. monodon* (Yamada et al., 1990; Pan and Chen, 2004).

Supplementation of experimental diets with *T. erecta* extract did not significantly alter the proximate composition of the practical diet because very small amounts were required to attain the desired concentrations in the feeds (less than 0.5% of the total ingredients). The same results were reported by Arredondo-Figueroa et al. (2003) and Flores et al. (2007). This is important in determining the commercial application of *T. erecta* supplements. The slight decrease in total carotenoid content of the pellets after drying may have resulted from the friction applied when the pellets were extruded. Synthetic and natural astaxanthin, commonly used in aquaculture diets, are obtained industrially and highly marketable. Extracts of marigold flowers are also industrially produced and, given the advantages demonstrated in this study, it is possible that they are a viable option to be evaluated in commercial practice.

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